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Functional characterization of HIV-1 Nef mutants in the context of viral infection

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Abstract

Nef is an important pathogenesis factor of HIV-1 with a multitude of effector functions. We have designed a broad panel of isogenic viruses encoding defined mutants of HIV-1_{SF2} Nef and analyzed their biological activity in the context of productive HIV-1 infection. Analysis of subcellular localization, virion incorporation, downregulation of cell surface CD4 and MHC-I, enhancement of virion infectivity and facilitation of HIV replication in primary human T lymphocytes mostly confirmed the mapping of Nef determinants previously reported upon isolated expression of Nef. However, reduced activity in downregulation of CD4, infectivity enhancement and virion incorporation of a Nef variant (Δ12–39) lacking an amphipatic helix required for binding of a cellular kinase complex and the association of Nef with MHC-I/AP-1 suggested a novel role of this N-terminal motif. The SH3 binding motif of Nef was partially required for infectivity enhancement and replication but not for receptor downmodulation. In contrast to previous results obtained using other Nef alleles, non-myristoylated SF2-Nef was only partly defective when expressed during HIV infection and was present in HIV-1 particles. Importantly, incorporation of Nef into HIV-1 virions was not required for any of the tested Nef activities. Altogether, this study provides a broad characterization and mapping of multiple Nef activities in HIV-infected cells. The results emphasize that multiple activities govern Nef's effects on HIV replication and argue against a role of virion incorporation for Nef's activity as pathogenicity factor.

Keywords: HIV Nef; Mutant virus panel; Virion incorporation; Myristoylation; Cell surface receptor modulation; Infectivity; Replication

Introduction

The accessory gene product Nef is a critical determinant for the pathogenesis of the primate lentiviruses HIV-1, HIV-2 and SIV. This is illustrated by low levels of replication in vivo achieved by viruses lacking functional *nef* genes that lead to no or delayed disease progression (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995). Such a role for Nef as pathogenicity factor is further substantiated by a transgenic mouse model (Hanna et al., 1998) in which isolated expression of Nef causes AIDS like disease. Despite intense research efforts, the mechanisms by which Nef mediates its role as pathogenicity factor have remained obscure. Numerous activities of the viral protein, all exerted by protein interaction with cellular ligands, have been described (Arora et al., 2002; Geyer et al., 2001). In vivo, these activities are thought to mediate shielding of HIV-infected cells from the host's immune response and to directly optimize the multiplication of the virus. Immune evasion activities of Nef include the downmodulation of cell surface MHC class I and II molecules as well as the induction of apoptosis in bystander cells (Collins et al., 1998; Schwartz et al., 1996; Stumptner-Cuvelette et al., 2001; Xu et al., 1997, 1999). The optimization of the cellular environment for viral replication is achieved by the activation of signal transduction cascades leading to prolonged survival of infected cells and increased virus production (Fackler and Baur, 2002; Schrager and Marsh, 1999; Swingler et al., 2003; Wolf et al., 2001). Additionally, Nef triggers the downmodulation of

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cell surface CD4, which appears to have a profound impact on the fitness of viral progeny (Garcia and Miller, 1991; Glushakova et al., 2001; Lundquist et al., 2002). These influences lead to enhanced infectivity of HIV particles produced in the presence of Nef when assayed in a single round of replication and in increased replication kinetics over multiple rounds (Aiken and Trono, 1995; Miller et al., 1994; Schwartz et al., 1995; Spina et al., 1994). In surgery with the downmodulation of cell surface CCR5, removal CD4 also interferes with superinfection of productively infected cells (Michel et al., 2005). The individual activities of Nef appear to be largely independent of each other, and specific protein interaction surfaces have been mapped that mediate individual Nef activities (reviewed in Arold and Baur, 2001; Geyer et al., 2001). A common prerequisite to all Nef actions consists in its tethering to intracellular and plasma membranes, which depends on the N-terminal myristoylation of Nef. Possibly mediated by its association with the plasma membrane, 10–100 Nef molecules are incorporated into HIV particles (Bukovsky et al., 1997; Fackler et al., 1996; Welker et al., 1998). Whether the virion incorporation of Nef plays a role for its effects on viral replication remains to be defined.

While the molecular mechanisms of individual Nef activities are being studied intensively, their relative contribution to Nef's role as pathogenicity factor is difficult to address. Studies using Nef mutants defective in individual protein interactions allowed the correlation of some of its activities with enhanced virus replication and/or disease progression. However, direct evidence for a role of any individual Nef activity in lentiviral pathogenesis is missing, and it seems unlikely that all activities of Nef reported thus far will be relevant in vivo. Moreover, some of the in vitro phenotypes of Nef are a matter of controversy, a fact that is often attributed to the use of different alleles of the variable *nef* gene in a wide array of divergent cell lines. An additional major obstacle in revealing the pathologically relevant Nef activities consists in the use of experimental systems that rely on the in vitro overexpression of Nef in the absence of other HIV gene products. While many of Nef's effects have been observed in the context of HIV infection individually, few studies directly compared the molecular determinants in Nef for multiple of its biological activities in infected cells. Some of these studies exclusively mapped the Nef determinants for optimal HIV replication and CD4 T cell depletion (Aldrovandi et al., 1998; Stoddart et al., 2003). Others correlated effects of Nef on HIV-1 replication with Nef activities determined in overexpression systems of divergent cellular origin and used various Nef alleles instead of specific isogenic mutants (Glushakova et al., 2001) or only addressed a limited number of Nef activities in infected cells (Lundquist et al., 2002).

Here, we generated and broadly characterized a panel of isogenic viruses that expresses an array of established Nef mutants. These viruses were used to analyze Nef activities in the context of HIV infection. Our results provide evidence that CD4 downregulation correlates best with the positive effects of Nef on HIV replication in primary human T lymphocytes and reveal an unexpected role of an amphipatic helix in the N-terminal anchor domain of Nef for its activities in CD4 downregulation, infectivity enhancement and boosting of HIV replication. Furthermore, the study reveals that virion incorporation of Nef is dispensable for its activities in infectivity enhancement and optimization of HIV replication.

Results

Generation of an isogenic nef HIV-1 virus panel

We set out to generate a panel of isogenic HIV-1 proviral clones encoding for 15 established Nef mutants. We chose to use the *nef* gene from the HIV-1 strain SF2 since the corresponding Nef isoform has proven very active in all assays for Nef functions investigated thus far (Baur et al., 1994; Fackler et al., 2001; Keppler et al., 2005; Krautkramer et al., 2004). Given that the HIV-1SF2 virus does not replicate very efficiently due to difficulties of Env virion incorporation (Stamatatos and Cheng-Mayer, 1993), SF2 *nef* variants were introduced into the genetic background of the HIV-1 strain NL4-3 lacking the *nef* gene (Δ*nef*). It had been previously determined that SF2 Nef exerts key activities of Nef such as enhancement of virion infectivity and increase of virus replication in such a chimeric virus (Fackler et al., 2001; Rucker et al., 2004). The prototype chimera containing the HIV-1 NL4-3 provirus with the HIV-1 SF2 *nef* gene was designated NL4-3 SF2Nef or wild type (WT), the respective *nef* variants were all designated according to their amino acid changes in the encoded Nef protein. Nef mutants included in the panel were based on previous biochemical or structural mapping of protein–protein interaction or modification sites in Nef (Table 1). Two mutants were expected to impact on Nef's ability to associate with cellular membranes, which is thought to be vital for all of its activities. G2ANef lacks the myristoyl receptor glycine at position 2 and can therefore no longer be myristoylated by *N*-myristoyl transferase, a modification that mediates tethering of Nef to cellular membranes (Geyer et al., 2001; Hill and Skowronski, 2005). The R4A4 mutant lacks a cluster of basic amino acids described as contributing to membrane targeting of Nef (Welker et al., 1998). Another set of mutants was expected to affect Nef mediated intracellular sorting: the LLAA and EDAA mutants no longer recruit adaptor protein (AP) complexes or the catalytic subunit of V-ATPase (V1H), respectively, and are deficient in CD4 internalization (Bresnahan et al., 1998; Craig et al., 1998; Greenberg et al., 1998a, 1998b; Lu et al., 1998; Piguet et al., 1998), while NefE4A4 is blocked in the ability to associate with the PACS sorting adapter and does not downregulate MHC-I and CCR5 (Greenberg et al., 1998a, 1998b; Piguet et al., 2000; Michel et al., 2005). CD4 downregulation should also be prevented by deleting or mutating the presumed CD4 binding site in the Δ59–61 or W61A Nef mutants (Grzesiek et al., 1996; Lu et al., 1998; Mangasarian et al., 1999). Another set of mutants was designed to block individual protein interactions Nef employs to modify intracellular signal transduction. These include mutations that interfere with the association of Nef with SH3 domains of e.g. Vav or Hck (P76/79A: NefAxxA, V78A, R81A). Presumably reflecting the need for an SH3 interaction, these mutations also abrogate the association of Nef with active

Table 1
Characteristics of Nef variants used

Nef variant	Mutation	Loss of interaction with	Reported functional consequences
WT	HIV-1 SF2 Nef	–	Functional Nef
G2A	G2A	N-myristoyl transferase	Loss of membrane association, non-functional Nef
R4A4	R17/19/21/22A	Membranes?	Reduced membrane association
W61A	W61A	CD4	Defect in CD4 downregulation
E4A4	E66/67/68/69A	PACS	Loss of MHC-I and CCR5 downregulation
AxxA	P76/79A	Pak2, SH3 domains (e.g., Vav, Hck)	Defect in downregulation of MHC-I and CCR5 and Pak2 association
V78A	V78A	Pak2, SH3 domains	Defect in association with Pak2 activity
R81A	R81A	Pak2, SH3 domains	Defect in association with Pak2 activity
L116A	L116A	Pak2, Nef	Defect in association with Pak2 activity and Nef multimerization
LLAA	L168/169A	AP-1, -2, -3, V1H	Defect in CD4 downregulation and infectivity enhancement
EDAA	ED178/179AA	AP-1, -2, -3, V1H	Defect in CD4 downregulation and infectivity enhancement
E181Q	E181Q	c-Raf	Unknown
P73A	P73A	TCR zeta, Pak2	Loss of FasL upregulation and association with Pak2 activity
Δ12–39	Δ12–39	NAKC	Reduced Nef-phosphorylation and stimulation of Tat transactivation
Δ59–61	Δ59CAW61	CD4	Loss of CD4 downregulation
RRLL	R109/110L	Pak2, Nef	Defect in association with Pak2 activity and Nef multimerization

Pak2 (Fackler et al., 1999; Manninen et al., 1998; Renkema et al., 1999; Saksela et al., 1995; Sawai et al., 1995). The latter activity of Nef is also abrogated by mutations in the Pak interaction surface of Nef, that however also interfere with the multimerization of the viral protein (L116A, RRLL) (Liu et al., 2000; Manninen et al., 1998; Sawai et al., 1995). In an additional mutant (Δ12–39), an amphipatic helix was deleted that mediates the association of Nef with a second signal transduction module, the Nef-associated kinase complex (NAKC) (Baur et al., 1997) as well as the association with MHC-I and AP-1 molecules (Roeth et al., 2004). Finally, variants that have been reported to be disrupted in their interaction with TCR zeta chain as well as Pak2 activity (P73A) (Xu et al., 1999) or the c-Raf kinase (E181Q) (Hodge et al., 1998) were included in the study.

Expression and subcellular localization of Nef variants

First, virus stocks were generated by transfecting proviral DNA into 293T producer cells. Western Blot analysis of lysates from these producer cells revealed similar Gag processing patterns in all cases (Fig. 1A, upper panel). Transfected 293T cells but also infected MT4 T lymphocytes produced comparable amounts of p24, indicating that Nef does not impact on the efficacy of particle production in these cells. Thus, net effects of Nef on virus production are only observed in COS-7 cells or in the presence of dominant-negative variants of members of the Vav-Pak signaling cascade (Fackler et al., 1999, 2000; Lu et al., 1996). Given this comparable virus production, the seemingly lower levels of Gag on the left side of the membrane are most likely due to local variations in western transfer efficiency. Thus, Gag expression was generally comparable. Analysis of Nef expression revealed specific gene products for all constructs except the Δ*nef* control (Fig. 1, lower panel). Of note, the E4A4 and Δ12–39 Nef mutants migrated faster than the other Nef variants because of the charge changes or deletions introduced during the creation of these specific mutants. Somewhat reduced expression levels were observed for the E4A4, W61A, Δ12–39, Δ59–61 and RRLL mutants of Nef. To address the potential relevance of these variations in protein stability for our subsequent functional analysis in T lymphocytes, Nef expression levels were quantified relative to Gag expression in lysates from MT4 T lymphocytes infected with the individual viruses (Fig. 1B). This analysis revealed that most of the Nef variants with the notable exception of RRLL were expressed at levels comparable or higher than wt. Such increased expression levels were particularly pronounced for G2A Nef. Thus, expression levels of individual Nef variants may vary depending on the cellular environment, and for the virus panel analyzed here, protein instability could be excluded as a major reason for altered biological activity of individual Nef variants in infected T lymphocytes.

The subcellular distribution of Nef is regarded a critical parameter for its activity, but only limited information is available on the localization of Nef and its variants in HIV-infected cells. We therefore performed an indirect immunofluorescence analysis on TZM cells 36 h post-infection after staining of the cells with antibodies against Nef and CA (Fig. 2). TZM cells were used to correlate Nef's subcellular localization with its effect on infectivity enhancement (see Fig. 4). At this time point, most of the infected TZM cells displayed large syncytia, often containing more than 10 nuclei. Importantly, the overall distribution of CA and Nef was not markedly different in single cells or at earlier time points post-infection (data not shown). The subcellular distribution of CA was comparable independent of the virus used for infection and revealed the expected punctate cytoplasmic distribution with some accumulation at the plasma membrane. In contrast, Nef was found more predominantly enriched in the perinuclear areas of infected cells, with some but not all cells showing marked staining of the plasma membrane and intracellular vesicles. No obvious colocalization between Nef and CA was observed. When comparing the distribution of the various Nef mutants, G2ANef, presumably due to the loss of myristoylation, was distributed much more diffusely in infected cells than WT Nef and was also found within the nucleus. The E4A4 Nef variant could not be analyzed since the stretch of acidic residues mutated in this variant overlaps with the epitope of the monoclonal antibody that was used for immunostaining of Nef. For all other Nef

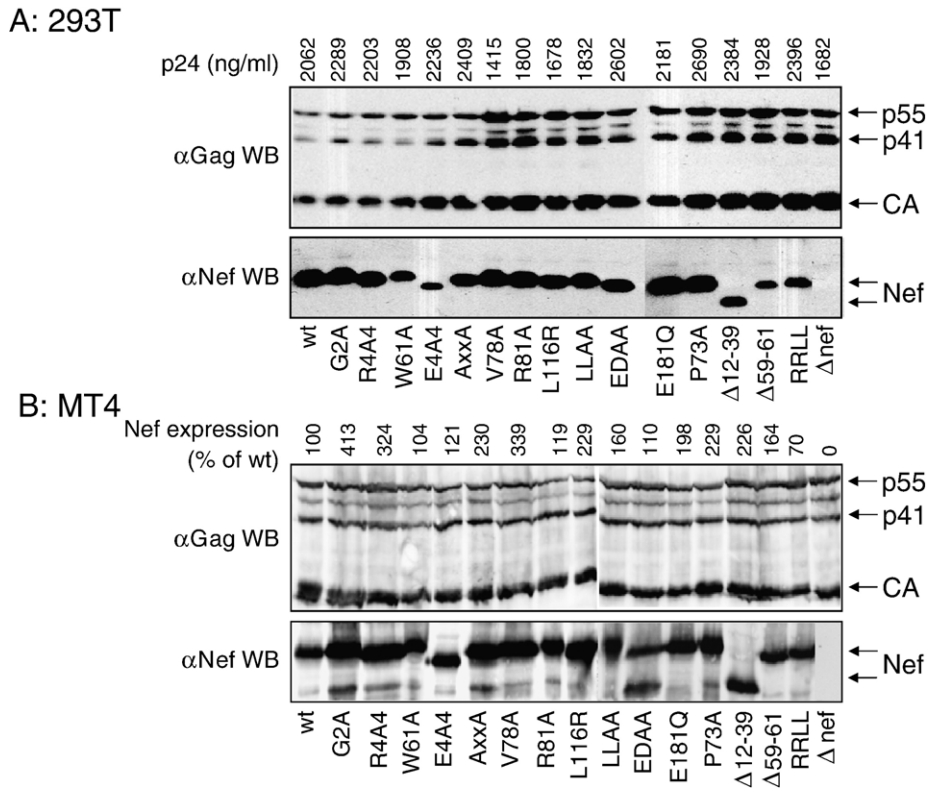

Fig. 1. Gag and Nef expression of the virus panel. (A) Western blot analysis of lysates from 293T cells 48 h post-transfection with the indicated proviral constructs for virus production. Numbers on top indicate the amounts of CA released in the cell culture supernatant. (B) Western blot analysis of lysates from MT4 cells infected with the indicated viruses. Cells were harvested and analyzed for the expression of Gag (upper panels) and Nef (lower panels). Arrows indicate unprocessed Gag (p55), processing intermediates (p41), CA or Nef, respectively. Detection and quantification of the CA and Nef signals were carried out using the LI-COR Odyssey imaging system and the relative Nef expression levels normalized to the respective CA signal are indicated above the lanes with wt arbitrarily set to 100%.

mutants, the subcellular distribution was not significantly changed relative to WT. This was surprising for the LLAA and EDAA variants as mutations in the c-terminal flexible loop of Nef are known to significantly alter its subcellular localization when overexpressed as a CD8-Nef fusion protein (Erdtmann et al., 2000; Janvier et al., 2003). However, such mutations do not affect the localization of Nef in cells transfected with Nef.GFP expression plasmids (Craig et al., 2000; Greenberg et al., 1998a, 1998b) or HIV proviral DNA (data not shown), indicating that this translocation is a specific property of CD8-Nef fusion proteins. Together, these results indicated that of all Nef variants tested in the virus panel, only the G2A mutation has a major impact on the overall steady state subcellular distribution of Nef. Furthermore, the presence of other HIV gene products in infected cells does not generally affect the localization of Nef. Subtle changes in Nef localization, however, may be masked by secondary effects induced by expression of the entire HIV genome in the infection context.

Myristoylation is not required for virion incorporation of HIV-1 SF2Nef

In a next step, we investigated the incorporation of Nef into virus particles produced from 293T cells. Cell culture supernatants were concentrated by ultracentrifugation through a sucrose cushion and normalized for their content of CA (Fig. 3A; upper panel). Western blotting for the presence of Nef revealed that significant amounts of Nef were detected in the WT preparations (bottom panel). Virion incorporation of Nef variants was analyzed in parallel and individual mutants were grouped into classes with near WT incorporation (++), intermediate (+) and undetectable incorporation (−), respectively (Fig. 3A). Surprisingly, loss of virion incorporation was only observed for the two Nef variants E4A4 and Δ12–39. In contrast, G2ANef, which was expected to lose virion incorporation because of the lack of N-terminal myristoylation, was detected in particle preparations at levels similar to WTNef. Most other Nef variants displayed approximately equal virion incorporation, while the L116R Nef mutant reproducibly showed clearly detectable, yet somewhat reduced virion incorporation efficiency. To confirm the unexpected properties of the G2A variant in virion incorporation, this analysis was repeated with two independent virus preparations (Fig. 3B). As seen before, robust amounts of G2ANef were detected in virus pellets from these infectious cell culture supernatants. To exclude that the previous mapping of Nef determinants for virion incorporation was the consequence of high levels of expression in transfected 293T cells, we analyzed cell culture supernatants of infected PM1 T cells (Fig. 3C). Nef and its G2A and E4A4 mutants were expressed to comparable levels while expression of the

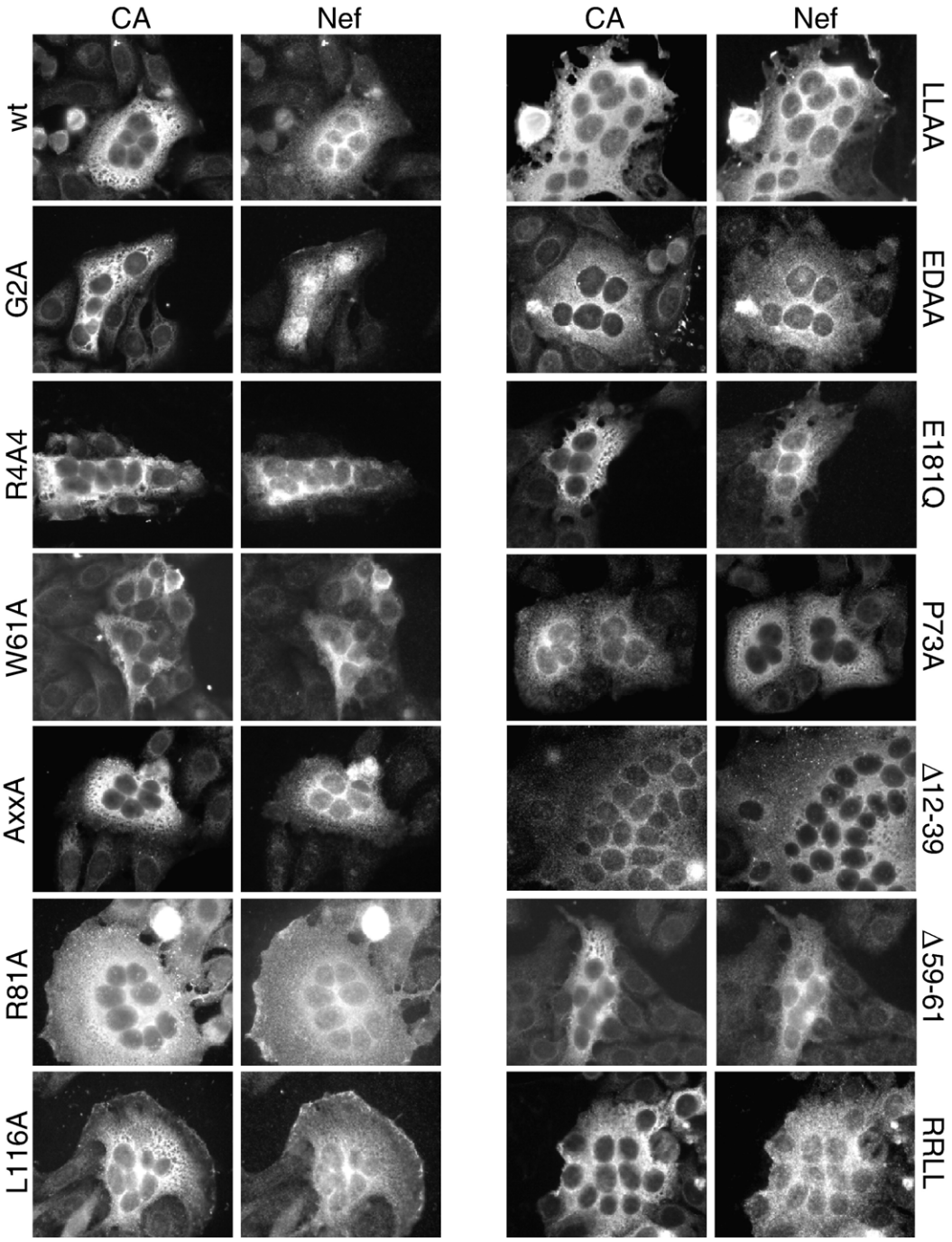

Fig. 2. Subcellular localization of Nef and CA in HIV-1 infected TZM cells. Immunofluorescence analysis of TZM cells infected with the indicated HIV-1 variants. 36 h post-infection cells were fixed and stained with antibodies specific for CA (left) and Nef (right). Shown are representative pictures of multinucleated syncytia, in which the overall distribution of CA and Nef was comparable to single infected cells.

Δ12–39 Nef mutant was markedly reduced. Analysis of virion pellets confirmed that G2ANef was incorporated in HIV-1 virions with an efficiency comparable to that of WT, while the E4A4 and Δ12–39 Nef variants were not detectable in these virion preparations. The observed virion incorporation of G2ANef is in contrast to previous reports on the loss of virion incorporation for a G2A variant of NL4-3 and HxB2 Nef proteins (Chen et al., 1998; Pandori et al., 1996; Welker et al., 1996). Using parallel transfection of isogenic proviral clones carrying G2A variants of either SF2 or NL4-3 Nef, we confirmed a strain specific difference where the SF2 mutant, but not the NL4-3 mutant, was incorporated efficiently (Fig. 4A). Although we observed some variations in the efficiency of G2A SF2Nef virion incorporation between experiments, quantification of the data presented herein revealed that, when normalized for the content of p24CA, G2ANef was present to levels of 94.7 ± 18% relative to wtNef. Notably, no cleavage of SF2 Nef by the HIV protease was detected, which had

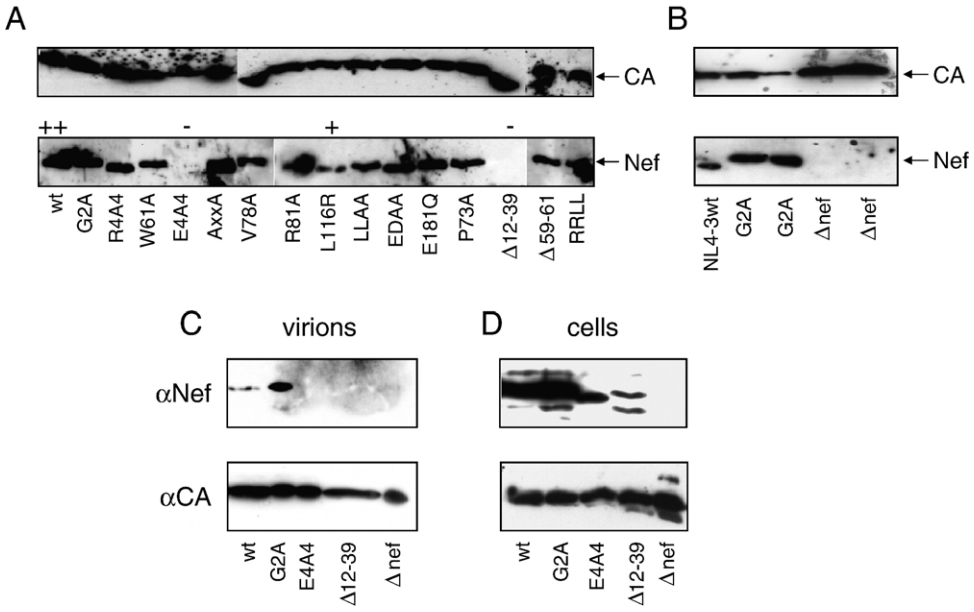

Fig. 3. Virion incorporation of Nef variants. (A, B) Western blot analysis of virus pellets generated by ultracentrifugation of cell culture supernatants from 293T cells that had been transfected with the respective proviral clones for virus production. Note that 5 ng or 100 ng total protein were loaded for the analysis of CA (upper panel) or Nef (lower panel), respectively. The data shown are representative for three independent virion preparations. (C, D) Analogous analysis with virus harvested from PM1 cells 3 days post-infection with the indicated viruses. Western blot analysis of virus pellets (C) and cell lysates (D), respectively.

been observed in several previous studies using different alleles (Pandori et al., 1996; Welker et al., 1996). Consistently, Nef cleavage was readily detected in parallel preparations of HIV-1 NL4-3 containing NL4-3 Nef (Fig. 4A), suggesting that the SF2Nef protein may be less susceptible to HIV protease cleavage or the cleavage product of SF2Nef may not be detected by our anti-Nef antibody. Since cleavage of Nef by the HIV protease occurs within intact virus particles and this cleavage was observed for NL4-3 Nef, these results also argue against a contamination with microvesicles as major source of the particle-associated Nef protein in our preparations. This was confirmed by an additional control where Nef was readily pelleted from supernatants of cells expressing the HIV-1NL4-3 SF2 provirus but not a SF2Nef. GFP fusion protein (Fig. 4B). To address whether the differential myristoylation requirement for virion incorporation of NL4-3 and SF2 Nef proteins reflects their membrane binding properties, membrane fractionation experiments were performed with infected Jurkat cells (Fig. 4C). Segregation of transferrin receptor (TfR) and 14-3-3 into the pellet (P) and cytosolic (S) cell fractions, respectively, served as quality control of the fractionation procedure. Expectedly, significant amounts of both Nef proteins (approximately 35%) were detected in the P fraction that contains membranes, cytoskeleton and protein aggregates. In contrast, the non-myristoylated variants of NL4-3 and SF2 Nef predominantly partitioned in the S fraction (approximately 94%). Together, these results identify previously unrecognized determinants for virion incorporation of SF2Nef, reveal that myristoylation is not a strict requirement for this process and demonstrate similar contribution of myristoylation to membrane binding of SF2 and NL4-3 Nef proteins.

Characterization of the determinants that mediate enhancement of virion infectivity by SF2Nef

To investigate the efficiencies by which the Nef variants represented in the virus panel augment HIV-1 infectivity in a single round of replication, TZM indicator cells were infected with 0.5 ng CA of the 293T derived virus stocks analyzed for virion incorporation in Figs. 3A and B. 36 h post-infection, HIV-infected cells were quantified on the basis of their characteristic blue staining (Figs. 5A and B). As expected, Δ*nef* virions were approximately 4-fold less infectious that HIV-1 WT particles (Aiken and Trono, 1995; Miller et al., 1994; Schwartz et al., 1995; Spina et al., 1994). Based on this result, virus particles with less than 30% infectivity relative to WT HIV-1 were considered deficient in Nef induced enhancement of infectivity. Such complete lack of Nef activity (−) was only observed for the Δ12–39 and the Δ59–61 variants. The R4A4 and E181Q mutations had no effect and resulted in virions with relative infectivities greater than 70% of wt HIV-1 and were thus considered as fully active (++). All other mutations caused intermediate phenotypes with partial Nef activity resulting in virions with relative infectivities ranging between 30% and 70% (+). Interestingly, the relative infectivity of G2A viruses ranged between 37 and 64% of that of WT and thus always scored intermediate in the infectivity assay. Furthermore, also the infectivity of HIV-1 particles produced from PM1 T lymphocytes was significantly increased by the G2A and E4A4 Nef but not by the Δ12–39 variant (Fig. 5C). Thus, several distinct interaction surfaces are required for Nef-mediated enhancement of virion infectivity and non-myristoylated Nef retains considerable activity in this assay.

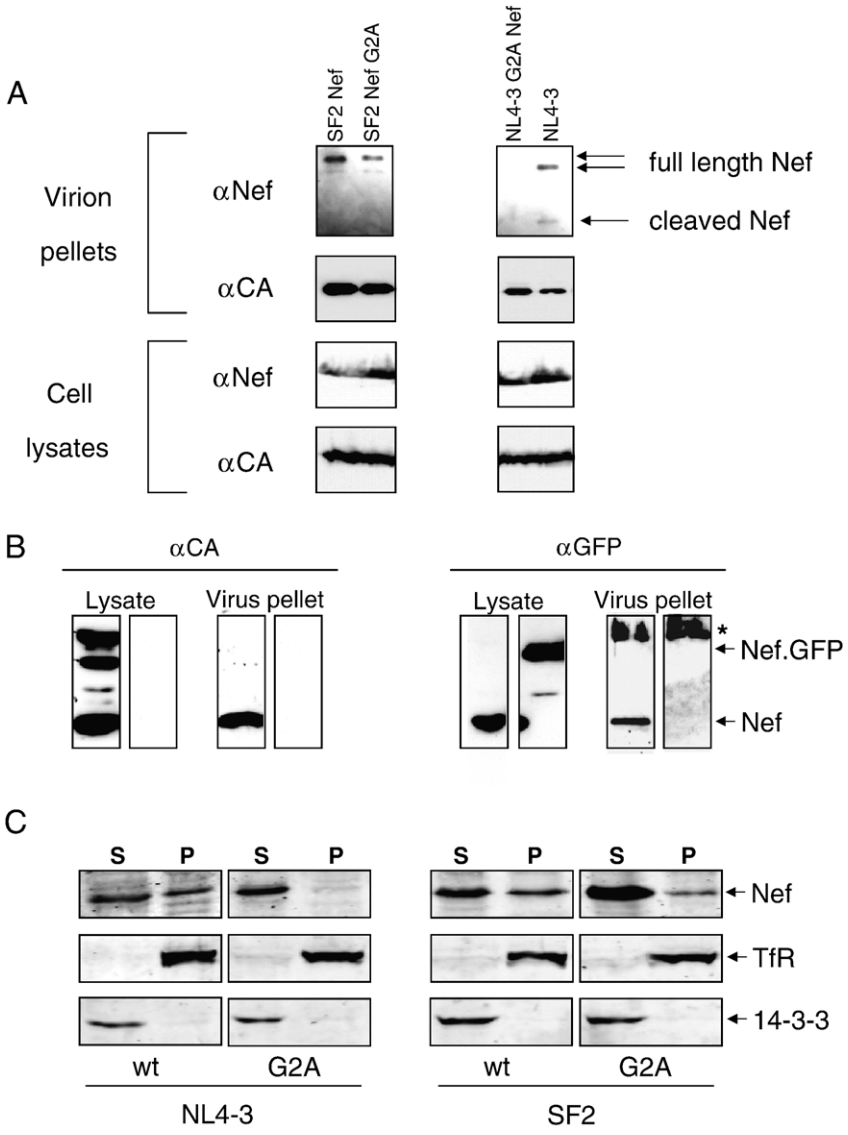

Fig. 4. Analysis of HIV particle preparations. (A) Comparison of virion incorporation of Nef proteins from the HIV-1 strains NL4-3 and SF2. 293T cells were transfected with HIV-1 NL4-3 proviral DNA encoding for NL4-3 or SF2 Nef proteins or their non-myristoylated G2A variants. Virion pellets and cell lysates were analyzed for the presence of CA and Nef by Western blotting. (B) 293T cells were transfected with proviral DNA or an expression plasmid for the SF2Nef.GFP fusion protein. Virion pellets and cell lysates were analyzed for the presence of CA (left panel) and Nef.GFP (right panel) by Western blotting. The asterisk denotes a non-specific band detected by the anti-GFP antibody in virus pellets. (C) Membrane fractionation of infected Jurkat T lymphocytes. Three days post-infection with viruses expressing the indicated Nef proteins, postnuclear extracts of Jurkat cells were fractionated into soluble (S) and pellet (P) fractions and analyzed by Western blotting for the distribution of Nef. Transferrin receptor (TfR) and 14-3-3 proteins were analyzed as markers for S and P fractions, respectively.

Analysis of CD4 downregulation in HIV-1 infected T lymphocytes

We next investigated steady state cell surface levels of CD4 in Jurkat T cells infected with the *nef* virus panel (Fig. 6). To define the experimental setup, cells were infected with WT and Δ*nef* viruses and stained for CD4 and intracellular CA 18 (early) and 48 (late) h post-infection (Fig. 6A). At the early time point, CD4 cell surface levels were not markedly reduced in Δ*nef* infected cells relative to the mock infected control. In contrast, WT-infected cells displayed a significant reduction in cell surface CD4, reflecting the action of Nef. Of note, analysis of the same cells at later time points revealed efficient CD4 downregulation in cells infected by both WT and Δ*nef* viruses, presumably reflecting the activity of Vpu and Env (Chen et al., 1996). Thus, Nef-specific effects of the virus panel were determined at 18 h post-infection (Fig. 6B). Quantification of CD4 cell surface levels relative to the mock infected control cells (100%) revealed that Δ*nef* infected cells maintained more than 75% of cell surface CD4, while these levels were reduced to less than 20% in WT-infected cells. Nef variants removing cell surface CD4 down to at least 30% were considered comparable to WT (++), an activity resulting in 30–70% cell surface presentation was scored as intermediate (+), and Nef variants that did not reduce cell surface CD4 below 70% were considered as inactive in this assay (−). As expected, the Nef variants thought to be unable to interact with the endocytic machinery (LLAA, EDAA) or with CD4 (Δ59–61) failed to

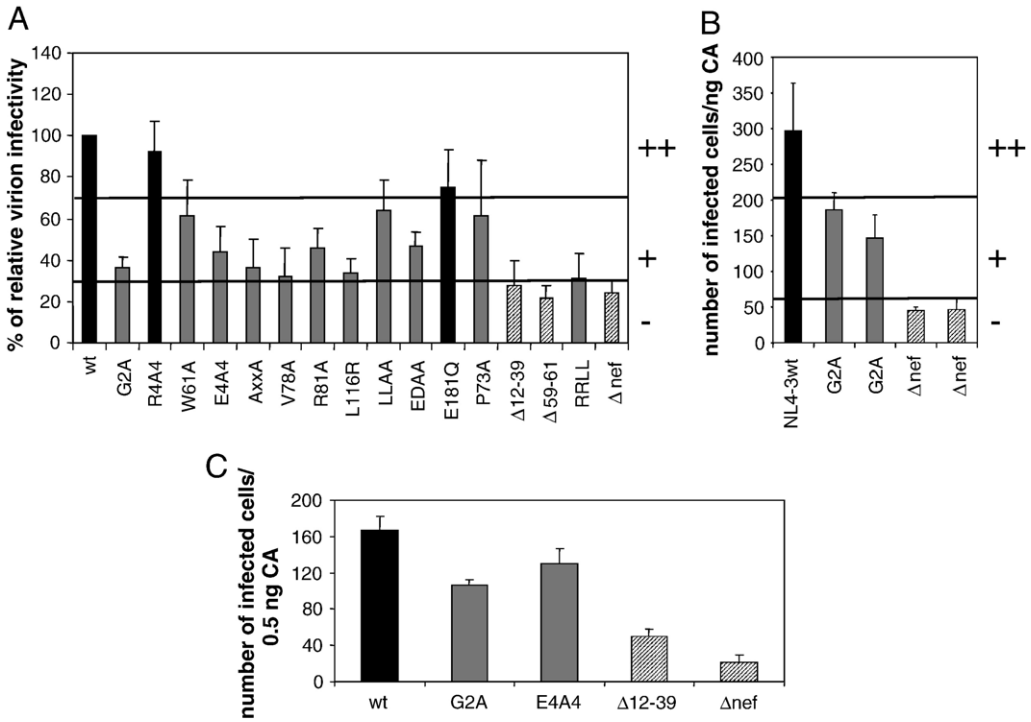

Fig. 5. Enhancement of virion infectivity by Nef variants. Single round of replication analysis on TZM cells. TZM cells were infected with 0.5 ng CA of the identical virus stocks that were analyzed for Nef virion incorporation in Fig. 3. 36 h post-infection, the cells were fixed, stained for β-galactosidase, and the number of blue cells was counted. Data represent average values from two independent experiments with triplicate measurements each with the indicated standard error of the mean. Depicted is the relative virion infectivity (number of blue cells per ng CA) with values for HIV-1NL4-3 NefSF2 (wt) arbitrarily set to 100% (A) or absolute numbers of infected cells per ng p24 (B, C). (A, B) 293T cell derived virus. Relative activity in enhancement of virion infectivity was grouped as "no Nef activity, (−) for values within 1 SD of Δnef (up to 30% activity), "intermediate Nef activity" (+) between 30–70% activity and "full Nef activity" (++) between 70–100% activity. (C) Virus derived from infection of PM1 T lymphocytes.

downmodulate the receptor. Of note, exclusively mutating W61 to A only partially affected Nef's ability to reduce cell surface CD4 levels, suggesting that this mutation is insufficient to disrupt the Nef–CD4 interaction. Interestingly, loss of CD4 downmodulation was also observed for the Δ12–39 variant. Three variants (G2A, L116A and RRLL) showed intermediate CD4 downregulation activity, while all other Nef variants efficiently removed the HIV entry receptor from the surface of virally infected cells.

Analysis of Nef-mediated MHC-I downregulation from the surface of HIV-infected T lymphocytes

With the same experimental setting effects of Nef on MHC-I surface presentation were monitored (Fig. 7). In this case, no significant difference was observed between early and late time points: at both times, downmodulation of MHC-I was readily observed in WT but not in Δ*nef* infected cells (Fig. 7A). Thus, under these experimental conditions, Nef is the only HIV gene product with significant effects on MHC-I cell surface levels. Reflecting the relatively less efficient downregulation of MHC-I by Nef as compared to CD4, MHC-I cell surface levels were only reduced to 58% in WT-infected cells relative to mock infected controls. Infection with the Δ*nef* control did not affect MHC-I cell surface levels. Of all Nef variants analyzed, only G2A, E4A4 and RRLL failed to downregulate MHC-I (relative levels higher than 80%), and only the Δ12–39 variant displayed an intermediate phenotype (58–80%). The majority of the tested Nef proteins efficiently downmodulated cell surface MHC-I. Interestingly, the LLAA, EDAA and Δ59–61 mutants, which no longer downregulated CD4, were more efficient than the WTNef protein in MHC-I downmodulation. It is likely that, in the absence of interaction of Nef with sorting proteins involved in CD4 trafficking or CD4 itself, the viral protein is more "available" for trapping MHC-I molecules. It was also surprising that the AxxA variant of Nef that was reported to be deficient in MHC-I downmodulation because of its inability to interact with SH3 domains (Greenberg et al., 1998a, 1998b; Manninen et al., 1998) retained actually a good activity on MHC-I. To exclude that the use of a pan anti-MHC-I antibody and the staining procedure for intracellular p24 affected the outcome of these experiments, we analyzed the effects of Nef in T1 lymphoid cells, which express the Nef-sensitive HLA-A2 molecule. Moreover, these cells express GFP under the control of the HIV-1 LTR indicating productive HIV infection by GFP fluorescence without the need for additional staining procedures. When analyzed 5 days post-infection, cell surface HLA-A2 was downmodulated to approximately 40% in cells infected with WT but not with Δ*nef* virus (Fig. 8). As observed in Jurkat cells, most of the Nef variants including the AxxA mutant displayed activities comparable to WTNef and the RRLL variant was fully defective in HLA-A2 downmodulation. The

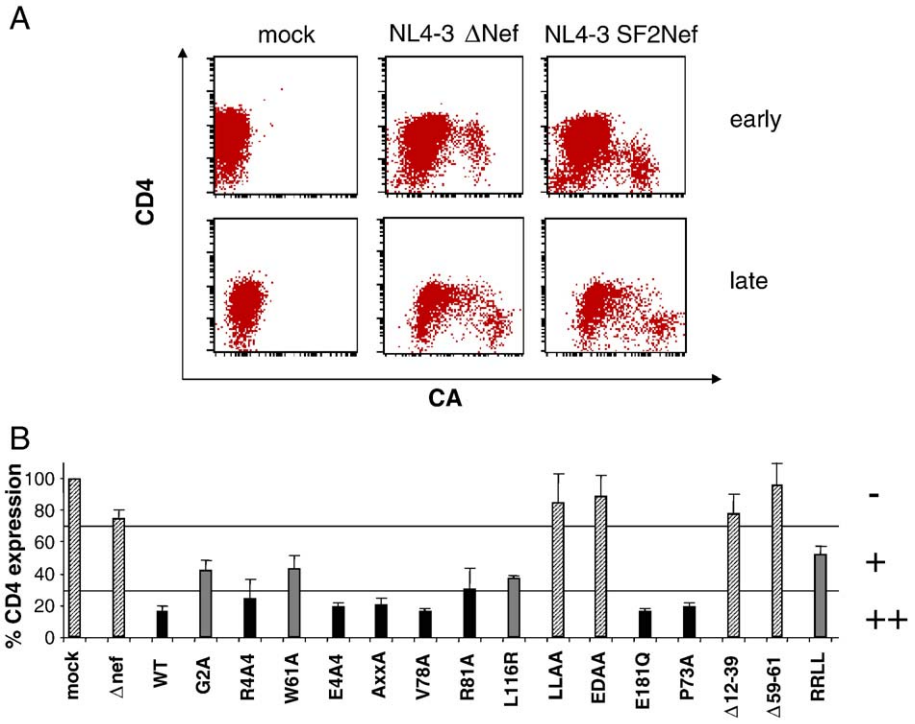

Fig. 6. CD4 surface levels in Jurkat cells infected with HIV-1 expressing various Nef mutants. (A) CD4 and CA expression in Jurkat cells infected with WT (NL4-3 SF2Nef) or ΔNef HIV (NL4-3 ΔNef). Jurkat cells were infected with viruses expressing the indicated Nef mutants. At 18 h ("early" panels) and 48 h ("late" panels) post-infection, cells were stained with anti-CD4 (surface staining) and anti-CA (intracellular staining) antibodies. (B) Activity of the various Nef mutants. CD4 surface levels of uninfected cells were set to 100%. CD4 surface expression with the panel of HIV-1 variants was determined 18 h after infection, within the fraction of CA+ cells. Data are mean ± SD of 3 independent experiments.

G2A and Δ12–39 Nef variants reduced HLA-A2 surface levels to approximately 60% of the control and thus displayed intermediate activity. Importantly, the E4A4 Nef protein that did not affect MHC-I surface levels in Jurkat cells was fully active in downmodulating HLA-A2 in T1 cells. Together, these results emphasize the need for the characterization of molecular determinants for Nef activity in cells acutely infected with HIV and pinpoint to potential cell type specific differences in the mechanism employed by Nef for cell surface receptor downmodulation.

Analysis of the determinants in Nef for the optimization of HIV-1 replication in primary human peripheral blood lymphocytes

As the final functional correlate we assayed the virus panel for replication in human peripheral blood lymphocytes (PBL) that had not been stimulated prior to infection. Following established procedures to reveal positive effects of HIV-1 Nef on viral spread (Miller et al., 1994; Spina et al., 1994; Welker et al., 1996), isolated PBMC were infected, cultured for 3 days and subsequently activated by PHA/IL-2. After 3 days of stimulation, PBL were cultured in the presence of IL-2, and virus replication was monitored by quantification of CA antigen in the cell culture supernatant. We first tested the replication kinetics of HIV-1 WT and Δ*nef* on PBL from various independent donors. As shown in Figs. 9A and B, PBL from most donors displayed a delayed replication curve for Δ*nef* relative to the WT control. In the experiments shown, a virus inoculum of 1 ng CA was used. Using higher doses of virus input accelerated virus spread, often masking the influence of Nef on HIV-1 replication (data not shown). Furthermore, some individual donors did not reveal significant differences between replication of WT and Δ*nef* HIV-1 even with low doses of virus input (data not shown). The PBL from the donor shown in Fig. 9B were used to assay virus replication for the entire virus panel in parallel. Fig. 9C presents CA concentrations in the medium at days 2 and 4 post-activation (black and grey bars, respectively). Shown is one experiment representative of three independent experiments each carried out in quadruplicates. Presumably due to the low virus input needed to reveal a Nef phenotype, individual wells did not produce detectable amounts of virus over the course of the experiment. These wells were excluded from the analysis, and the total number of productively infected wells is provided in Fig. 9C. Based on the CA production of wt and Δ*nef* viruses on day4, the panel was grouped as having full (++, 100–70%), intermediate (+, 70–30%) and no (−, below 30%) Nef activity. Variants such as E4A4 that could not be attributed with certainty to a specific activity group due to variability between experiments were scored as intermediate between the respective activity groups (see Table 2). Comparable overall grouping was obtained with PBL from an independent donor (data not shown). Based on this analysis, G2A, LLAA, EDAA and Δ12–39 Nef variants were identified as defective in boosting HIV-1 replication in primary T cells. This effect was most

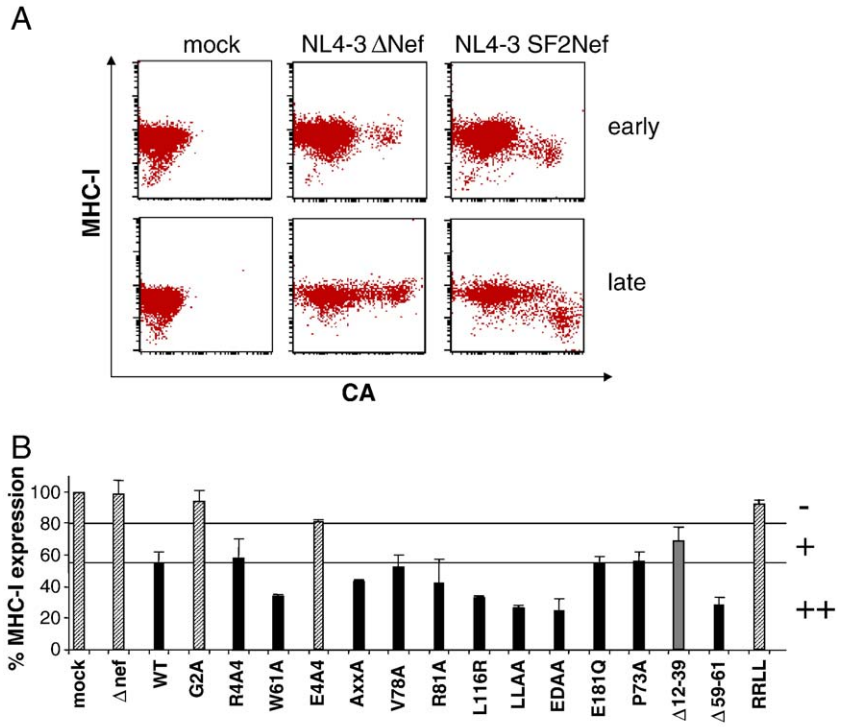

Fig. 7. MHC-I surface levels in Jurkat cells infected with HIV-1 expressing various Nef mutants. (A) MHC-I and CA expression in Jurkat cells infected with WT (NL4-3 SF2Nef) or ΔNef HIV (NL4-3 ΔNef). Jurkat cells were infected with viruses expressing the indicated Nef mutants. At 18 h ("early" panels) and 48 h ("late" panels) post-infection, cells were stained with anti-MHC-I (surface staining) anti-CA (intracellular staining) antibodies. (B) Activity of the various Nef mutants. MHC-I surface levels of uninfected cells were set to 100%. MHC-I surface expression with the panel of HIV-1 variants was determined 18 h after infection, within the fraction of CA+ cells. Data are mean ± SD of 3 independent experiments.

pronounced for the LLAA and EDAA Nef mutant viruses that replicated even less efficiently than Δ*nef*. Re-examination on PBL from a different donor consistently revealed replication comparable to HIV-1Δ*nef* (data not shown). In contrast, mutation of R4, W61, E4, V78 and L116 did not have significant impact on Nef's ability to enhance viral spread in

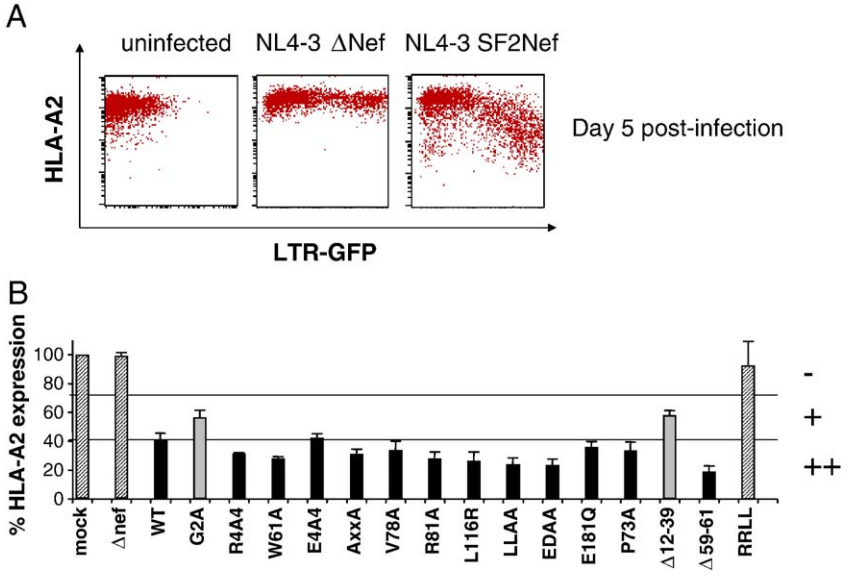

Fig. 8. MHC-I A2 surface levels in CEM cells infected with HIV-1 expressing various Nef mutants. (A) MHC-I and CA expression in CEM cells infected with WT (NL4-3 SF2Nef) or ΔNef HIV (NL4-3 ΔNef). CEM cells were infected with viruses expressing the indicated Nef mutants. At 5 days post-infection HLA-A2 surface levels and LTR driven GFP expression were analyzed by flow cytometry. (B) Activity of the various Nef mutants. HLA-A2 surface levels of uninfected cells were set to 100%. HLA-A2 surface expression with the panel of HIV-1 variants was determined 5 days post-infection, within the fraction of GFP+ cells. Data are mean ± SD of 2 independent experiments.

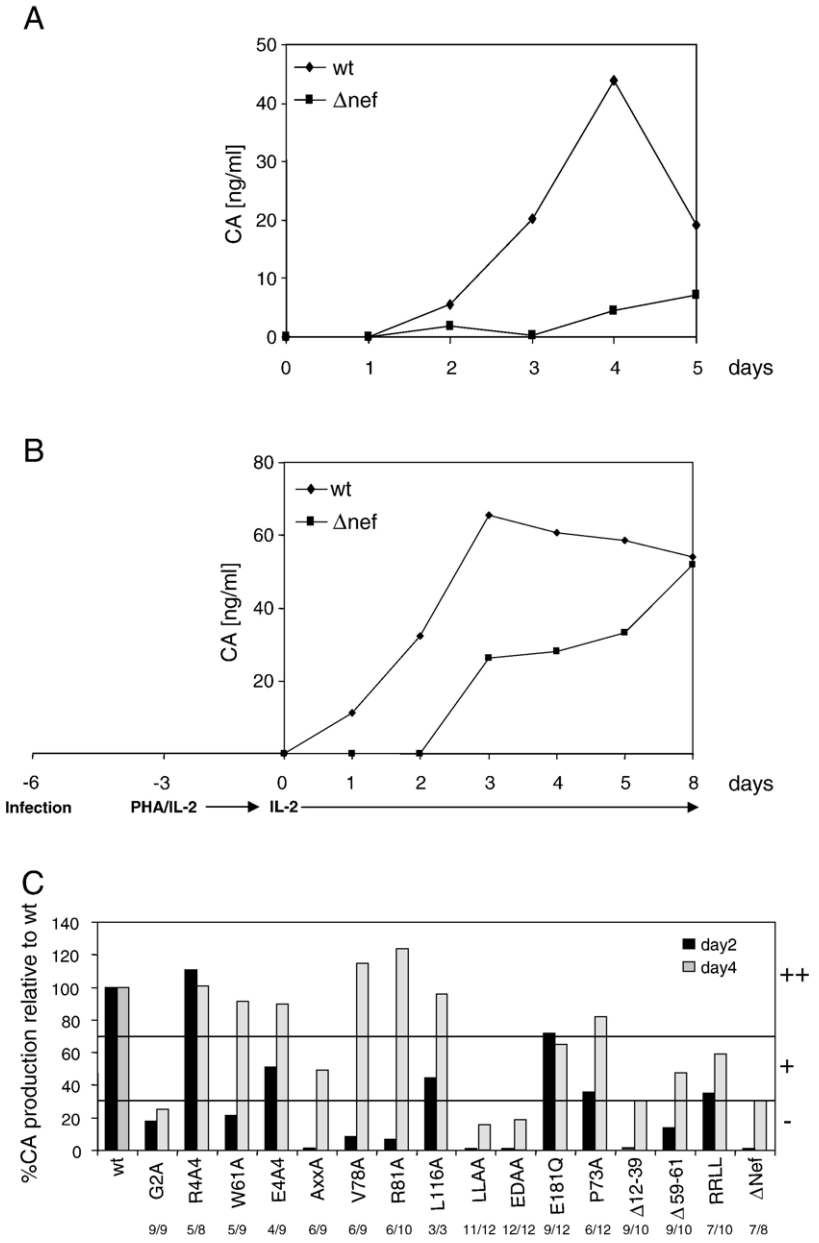

Fig. 9. Effects of Nef variants on HIV-1 replication in PBL. HIV replication was measured in 96-well plates on 1×10^5 PBL per well and 1 ng CA virus input. Freshly isolated, non-activated cells were infected (day −6) for 3 days and subsequently activated by PHA/IL-2 for 3 days. Starting from day 0, cells were kept in the presence of IL-2, and cell culture supernatants were collected each day to monitor CA production. (A, B) Representative replication kinetics of wt (diamonds) and Δnef (squares) HIV-1 on PBL from two different donors. CA values represent the average from quadruplicate infections performed in parallel. (C) Relative CA production of the isogenic *nef* virus panel on day 2 (black bars) and on day 4 (grey bars). CA values for wt were arbitrarily set to 100%. Shown is relative CA production form individual infections representative of the average result obtained in three independent experiments performed in quadruplicates. Relative CA production on day 4 was used to group Nef variants as "no Nef activity (−, up to 30% relative activity, white bars), "intermediate Nef activity" (+, 30–70% relative activity) and "full Nef activity" (++, 70–100% relative activity). Numbers below the bars indicate how many of all individual values tested resulted in the grouping represented here.

PBL while the remaining Nef variants displayed intermediate activity. As Nef has also been reported to increase the TCID50 determined on activated PBMC four donor pools (Miller et al., 1994; Sinclair et al., 1997), TCID50 values were also determined for our virus stocks. While this analysis confirmed an increase of HIV-1 infectivity on human primary T lymphocytes, results from independent experiments gave heterogeneous results and did not allow a firm quantification of the contribution of individual Nef protein interaction sites to virus replication (data not shown). Together, distinct protein interaction sites in Nef were identified that are required for optimal replication of HIV-1 in primary human T cells.

Discussion

This study presents the functional characterization of the HIV pathogenicity factor Nef in HIV-infected cells using a broad panel of defined Nef mutants in an isogenic background.

Table 2
Summary of relative Nef activities in HIV-1 infected cells

Nef variant	Loss of interaction with	CD4 downregulation	MHC-I[a] downregulation	Infectivity enhancement	Virion incorporation	PBL replication
WT	–	++	++/++	++	++	++
G2A	NMT	+	–/+	+	++	–
R4A4	membranes?	++	++/++	++	++	+/++
W61A	CD4	+	++/++	+	++	+/++
E4A4	PACS	++	–/++	+	–	+/++
AxxA	SH3 domains	++	++/++	+	++	+
V78A	Pak2, SH3 dom.	++	++/++	+	++	++
R81A	Pak2, SH3 dom.	++	++/++	+	++	+/++
L116A	Pak2, Nef	+	++/++	+	+	++
LLAA	AP-1,-2, -3, V1H	–	++/++	+	++	–
EDAA	AP-2, V1H	–	++/++	+	++	–
E181Q	C-Raf	++	++/++	++	++	+
P73A	TCR zeta, Pak2	++	++/++	+	++	+/++
Δ12–39	NAKC	–	+/+	–	–	–
Δ59–61	CD4	–	++/++	–	++	+
RRLL	Pak2, Nef	+	–/–	+	++	+
Δnef	all	–	–/–	–	–	–

[a] MHC-I in Jurkat/HLA-A2 in T1.

All Nef variants included in this study expressed proteins of the expected size but to somewhat variable levels. These variations were cell type dependent: while several Nef variants were less stable than wt in 293T cells, almost all Nef variants expressed to higher levels than wt in T lymphocytes. Moreover, Nef variants such as W61A, Δ59–61 or E4A4 that were expressed to lower levels in 293T cells still displayed full biological activity in select Nef functional assays, indicating that the observed variations in expression levels are unlikely to account for most of the functional differences observed between individual Nef variants. The analysis of Nef's subcellular localization and its effect on the cell surface density of CD4 and MHC-I molecules served as basic functional characterization of the Nef virus panel. These investigations did not include the analysis of signal transduction properties of Nef that can be monitored, e.g., by its ability to associate with and activate the cellular Pak and Lck kinases (Baur et al., 1997; Fackler et al., 2000; Renkema et al., 1999; Witte et al., 2004). Such studies depend on prior immunoisolation of Nef. Since the aim of this study was the characterization of Nef in its natural proviral context, no epitope tags were added to the Nef proteins in order to prevent all putative modulation of Nef's biological activity. Due to the lack of specific antibodies that immunoisolate all Nef variants tested here with comparable efficiency without interfering with its kinase association, the analysis of these signaling events was precluded. The analysis of CD4 surface levels in infected cells gave results that are consistent with the prevailing idea that Nef targets the cytoplasmic tail of CD4 with its hydrophobic pocket and routes the receptor to degradation via interactions with the cellular endocytosis machinery that are mediated by its c-terminal flexible loop (Lama, 2003). The results obtained also confirm the mechanistic segregation of CD4 and MHC-I downmodulation in HIV-infected cells.

Conversely, our analysis of Nef-mediated downregulation of MHC-I in the context of HIV-1 infection yielded several noteworthy insights. First, Nef was confirmed as the only HIV gene product with an appreciable effect on MHC-I cell surface exposure. Second, Nef reduced overall MHC-I or HLA-A2 cell surface density to only 40% in infected T lymphocytes in this study. Of note, similarly moderate levels of MHC-I down-modulation can be sufficient to protect HIV-infected cells from CTL lysis (Tomiyama et al., 2005). Third, the mapping of Nef determinants in the infection context varied from previous results using Nef overexpression systems. Surprisingly, disruption of the PxxP motif in Nef had no appreciable effect on MHC-I downregulation, indicating that the proposed role for the interaction of Nef with SH3 domains in this process (Blagoveshchenskaya et al., 2002; Greenberg et al., 1998a, 1998b) may not be relevant in HIV-infected T lymphocytes. This is consistent with recent results showing that the proline at position 78 rather than the two central prolines of the SH3 binding motif in Nef are critical for Nef-mediated MHC-I downregulation (Yamada et al., 2003), and that this Nef activity is indeed uncoupled from its ability to bind SH3 domains (N. Casartelli and M. Doria, personal communication). On the other hand, residues 12–39, Nef myristoylation and the di-arginine motif were essential for full Nef activity on MHC-I. Interestingly, an N-terminal amphipatic helix missing in the Δ12–39 Nef mutant was recently shown to assemble a complex containing MHC-I and AP-1 that is critical for Nef's down-modulating ability (Roeth et al., 2004; Williams et al., 2005). The requirement for myristoylation likely mirrors the need for efficient membrane attachment of Nef for receptor down-modulation. The RRLL Nef mutant classically served as a loss-of-function variant for the association of Nef with Pak activity (Sawai et al., 1996). Since Nef mutants such as AxxA that also do not associate with active Pak2 (Fackler et al., 1999; Manninen et al., 1998) downregulated MHC-I with full efficiency, involvement of Pak association can be excluded. Of note, the RRLL Nef variant did not reach full activity in any of the assays performed here except virion incorporation. This may reflect either broad unspecific effects, the slightly reduced stability of this protein or specific interference with Nef multimerization caused by the RRLL mutation (Liu et al.,

2000). Finally, the acidic cluster that mediates the interaction of Nef with PACS was required for MHC-I downmodulation in Jurkat but not in T1 cells. This emphasizes the possibility that Nef employs diverse molecular mechanisms for downregulation of MHC-I depending on the cellular context (Kasper and Collins, 2003).

One surprising result of our analysis was the fact that myristoylation was not absolutely required for most activities of SF2Nef and in particular not for its incorporation into HIV-1 virions. Such residual activity of non-myristoylated Nef likely reflects its relatively high levels of expression, and the fact that myristoylation is not the only determinant for membrane anchorage of Nef (Bentham et al., 2006). In contrast, virion incorporation of Nef from HIV-1 NL4-3 and HxB2 was reported to strictly require myristoylation (Bukovsky et al., 1997; Chen et al., 1998; Welker et al., 1998). Our results suggest that this discrepancy reflects allelic differences between Nef proteins from the HIV-1 strains NL4-3 and SF2 that is however, not determined by differential contributions of the myristoyl moiety to overall membrane binding. Consistent with such allelic differences, a mutation in the N-terminal basic cluster of Nef (R4A4), that was previously identified as one determinant for membrane binding of NL4-3Nef (Welker et al., 1998), does not impact on the activity of SF2Nef in HIV-1 infected cells (this study) or its membrane association (S.I. Giese and O.T. Fackler, unpublished). While the reason for this differential behavior remains to be determined, it may also account for the differential activity during HIV-1 entry in some cell systems (Fackler and Peterlin, 2000). In contrast to myristoylation, the amphipatic N-terminal helix that serves for the assembly of the NAKC signalosome and the association of Nef with the AP-1/MHC-I complex (Baur et al., 1997; Roeth et al., 2004; Williams et al., 2005) as well as the stretch of acidic amino acids mediating Nef's interaction with PACS (Piguet et al., 2000) were identified as novel determinants for virion incorporation of Nef. The molecular basis for the requirement of these motifs is uncertain. One possible model of virion incorporation of Nef predicts this to be a consequence of its association with the plasma membrane at budding sites. The rough correlation between determinants for Nef virion incorporation and its ability to downregulate cell surface MHC-I observed could indicate that Nef utilizes a common pathway for retention of MHC-I molecules during biosynthetic transport and its own translocation to budding virions. However, the Nef mutants lacking virion incorporation were fully functional in assays such as CD4 downmodulation that strictly depend on membrane association of Nef and did not display significant changes in their subcellular localization. An alternative view holds that Nef is incorporated into HIV particles by virtue of its physical interaction with GagPol (Costa et al., 2004). The motifs in the N-terminus of Nef may therefore be involved in the interaction of Nef with HIV structural proteins. Additionally, the analysis of this virus panel allowed us to draw conclusions on the role of virion Nef in the context of HIV replication. Several variants of the panel were efficiently incorporated into HIV particles yet failed to support virus replication in PBL, and the E4A4 Nef variant optimized HIV spread despite the lack of detectable virion incorporation. Similarly, no strict correlation was observed between the amounts of virion incorporated Nef and its ability to augment the infectivity of HIV-1 particles. This is seemingly at odds with recent reports from intravirion fusion and natural endogenous reverse transcription experiments, suggesting that virion Nef may be instrumental for Nef-mediated enhancement of virion infectivity and that localize the effect of Nef to a post-entry step in HIV target cells (Aiken and Trono, 1995; Cavrois et al., 2004; Khan et al., 2001, 2005; Schwartz et al., 1995; Tobiume et al., 2003; Zhou and Aiken, 2001). However, the results of these studies could also be explained by modifications of virion components imprinted by Nef during virus particle production. Together, our results strongly argue against an essential role of virion Nef in the replication of HIV-1 in PHA-activated PBL and in Nef-mediated enhancement of virion infectivity.

Our results also reveal a requirement for an N-terminal amphipatic helix for Nef-mediated CD4 downregulation and enhancement of virion infectivity as well as for Nef's virion incorporation. The interpretation of this result is complicated by the apparently reduced stability of the NefΔ12–39 variant in some cells. However, in the context of T lymphocyte infection, NefΔ12–39 was well expressed and retained partial MHC-I downregulation activity, a Nef function that requires high Nef expression levels (Liu et al., 2001). In contrast, this Nef variant failed to downmodulate cell surface CD4, which can readily be induced by low levels of functional Nef, suggesting that the defects observed reflect at least in parts specific alterations of Nef activity introduced by the 12–39 deletion. Such a role would be consistent with one report on the role of amino acid positions 29 and 36 in Nef from HIV-1 NA7 in Nef-mediated CD4 downregulation (Iafrate et al., 1997), while deletion of this helix had no effects in other studies using Nef overexpression (Mangasarian et al., 1999; Williams et al., 2005). Mechanistically, this helix serves as interaction site of the NAKC signalosome as well as a protein complex containing AP-1 and MHC-I and was previously reported to contribute to the positive effects of Nef on HIV-1 replication in Jurkat cells (Baur et al., 1997; Roeth et al., 2004; Williams et al., 2005). The data presented here confirm and extend these results to primary human T cells. In line with these findings, this domain was recently reported to contribute to the pathogenic potential of Nef in transgenic mice (Hanna et al., 2004). Whether these functional consequences of deletions in the N-terminus of Nef reflect the direct involvement of NAKC-mediated signaling, AP-1-mediated intracellular protein transport or other effects of this protein interaction surface warrants further investigation.

One important aspect of this study lies in the evaluation of HIV-1 replication in PBL stimulated with PHA post-infection as read-out for positive effects of Nef on viral spread. It has been described more than a decade ago that Nef provides detectable advantage to HIV replication in primary human T cells only when they are activated following infection but not when previously activated cells are used (Miller et al., 1994). This experimental set-up, that was also employed here, was subsequently used as correlate for Nef activity by many investigators (e.g., Rucker et al., 2004; Spina et al., 1994;

Welker et al., 1998), and our results confirm the basic observation of Nef-mediated enhancement of HIV-1 replication in that system. However, the accurate quantification of activities of a large virus panel proved difficult in our hands. Since low doses of virus input have to be used to reveal any effect of Nef, infections are carried out near the threshold for a productive infection, resulting in individual infections that remain non-productive. Furthermore, the variability in CA production between individual parallel infections together with the relatively subtle overall effect of Nef in this system did not allow solid quantification of intermediate Nef activities. Thus, while this experimental set-up serves well to detect loss-of-function mutations in Nef, it does not seem suitable for the quantification of partial Nef activities. Within these limitations, the rough grouping implemented here revealed that all Nef variants without detectable activity in boosting HIV-1 spread were defective in the downregulation of cell surface CD4. Similarly, all Nef variants without CD4 downregulation activity failed to enhance HIV-1 replication in PBL, with the exception of NefΔ59–61 that retained weak activity. No close correlation could be established for optimal HIV replication in PBL and the other Nef activities tested, indicating that these Nef effects may not be directly involved in HIV-1 replication in this experimental system. These results confirm and extend the previous notion that downmodulation of CD4 by Nef contributes to its effects on HIV replication (Glushakova et al., 2001; Lundquist et al., 2002). This may reflect the role of Nef-mediated CD4 downregulation in interference with negative effects of CD4 on HIV-1 infectivity and particle release as well as superinfection of already productively infected cells (Benson et al., 1993; Lama et al., 1999; Michel et al., 2005; Ross et al., 1999) but also other, CD4-independent effects mediated by the same protein interaction surfaces in Nef (Madrid et al., 2005). While these results are consistent with a requirement for Nef-mediated CD4 downmodulation for optimal viral replication, the intermediate activities of Nef variants with full CD4 downregulation capacity (AxxA, E181Q, RRLL) clearly indicate the existence of other mechanisms utilized by Nef to facilitate HIV-1 spread in primary human T cells. Interestingly, infection of PBL from some donors did not reveal any Nef phenotype (not shown). Revealing the cause for this donor variability would provide valuable information on these additional mechanisms. Of note, alternative primary cell systems have been reported recently that reveal more pronounced effects of Nef on HIV replication. These include cocultures of T cells with immature dendritic (Fackler et al., 2001; Petit et al., 2001) or vascular endothelial cells (Choi et al., 2005), ex vivo cultures of human tonsils (Glushakova et al., 2001) and T cells activated by SEB and mitomycin C-killed DCs (Lundquist et al., 2002). Thus, the nature of the activation stimulus appears to determine to which degree Nef is required for optimal HIV replication in primary human T cells. However, it is unclear at this point if all these systems monitor similar aspects of Nef biology and how accurately they reflect Nef's enhancement of virus replication in vivo. This question warrants further in depth analysis of the molecular mechanisms employed by Nef to augment HIV replication in such primary cell systems. The virus panel of isogenic Nef variants presented in this report will be a valuable tool for these analyses.

Materials and methods

Cells and reagents

293T and TZM (JC53BL) cells were maintained in DMEM high medium supplemented with 10% fetal calf serum, L-glutamine and antibiotics. Jurkat, PM1, C8166, MT4 and T1 (CEMx721.174 cells carrying an HIV-LTR-GFP cassette and expressing HLA-A2) lymphoid cells were cultured in RPMI 1640 medium supplemented accordingly. RPMI for PBMC cultures was supplemented with 10% FCS, 0.1% Glutamax (Invitrogen, Karlsruhe, Germany), 0,1% 1 M HEPES pH 7,4 and antibiotics.

Expression plasmids

Expression plasmids for GFP fusion proteins of HIV-1 SF2 Nef and its mutants were described previously (Keppler et al., 2005). Isogenic proviral constructs expressing various *nef* genes were constructed based on the pNLblue vector that contains the full-length proviral DNA of HIV-1 NL4-3 in the blueskript 2.9 vector backbone without flanking cellular genomic DNA. A single NotI restriction site was eliminated by digest, fill-in and religation to create pNLblueΔMCSNot. From this construct, a StuI/XhoI fragment was excised and replaced with the corresponding fragment from the pNL4-3Δmyr proviral clone (Welker et al., 1998), that carries a NcoI restriction site at the 5′ end of *nef*, resulting in the construct pNLblue Nco *nef*. Using mutagenic PCR, a NotI restriction site was introduced at the 3′ end of *nef* to create the pNLblue Nco *nef* Not vector. All *nef* variants from HIV-1SF2 described in this study were PCR amplified from DNA templates provided by the consortium "The HIV-1 pathogenicity factor Nef as a novel therapeutic target" (project QLK2-CT-2000-01630 in the fifth EC Framework Programme) or described previously (Keppler et al., 2005; Krautkramer et al., 2004) with primers introducing NcoI and NotI sites 5′ and 3′ of the *nef* amplicon, respectively, and were subcloned in the NcoI/NotI digested pNLblue Nco *nef* Not vector. The complete nucleotide sequence of all *nef* inserts of these proviral clones as well as of PCR amplicons from cells infected with the respective virus stocks was verified by sequencing of both DNA strands.

Western blotting

For Western blot analysis, samples were boiled in SDS sample buffer, separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Protein detection was performed following incubation with appropriate first and secondary antibodies using the super signal pico detection kit (Pierce, Bonn, Germany) according to the manufacturer's instructions. CA and Nef were detected using the polyclonal rabbit serum CA1 (Muller et al., 2004) and the polyclonal sheep serum Arp444 (Coates et al., 1997), respectively. For quantification of

Western blot signals, secondary antibodies coupled to the fluorophor Alexa 680 (Invitrogen) were used to detect and quantify the signals using a Odyssey imaging system (LI-COR Biosciences, Bad Homburg, Germany).

Virus stocks and virion infectivity

Virus stocks were generated by transfection of proviral HIV plasmids into 293T cells by the Ca-phosphate method. Two days after transfection, culture supernatants were harvested. The CA concentration of concentrated stocks was determined by CA antigen enzyme-linked immunosorbent assay (ELISA) as described (Muller et al., 2000). The relative infectivity of HIV-1 particles was determined by CA ELISA and a standardized 96-well TZM blue cell assay as described (Keppler et al., 2005). Briefly, infections were carried out in triplicates with 0.5 ng CA input virus. 36 h post-infection, cells were fixed, stained for β-galactosidase, and the number of blue cells was determined by microscopy. For virus production from PM1 T lymphocytes, 1×10^6 cells were infected with 3×10^5 infectious units (determined on TZM cells), and culture supernatants were harvested 3 days post-infection. For the infection of MT4 T lymphocytes, infected C8166 cells displaying pronounced syncytia were cocultured with MT4 cells, and cells were harvested after multiple serial passages.

Subcellular fractionation

Subcellular fractionation was performed using slight modifications of a previously described protocol (Alland et al., 1994). In brief, 1×10^7 Jurkat cells were infected with 600 ng of the indicated HIV-1 virus stocks by virus by spin infection at 20×*g* for 1.5 h. 3 days post-infection, the cells were resuspended in hypotonic buffer (10 mM Tris, pH 7.4, 0.2 mM $MgCl_2$, protease inhibitor cocktail) and homogenized with 50 pipetting steps. Postnuclear supernatants were adjusted to 0.25 M sucrose and 1 mM EDTA and centrifuged at 100,000×*g* for 50 min. Volumes of pellets (P100) and supernatants (S100) were equalized using RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.2, 1% Triton-X 100, 0.1% SDS). 6× Sample buffer was added, and aliquots were analyzed by Western blotting using antibodies against Nef, TfR (mAb H68.4, Zymed Laboratories Inc., South San Francisco, CA) and 14-3-3 γ (C-16, Santa Cruz Biotechnology, Heidelberg, Germany).

Virion incorporation

For the analysis of virion incorporation of Nef, 400 μl of cell culture supernatants containing infectious virions was placed on top of 80 μl 20% sucrose solution. After ultracentrifugation (TLA45 rotor, 44,000 rpm, 4 °C, 60 min), the pellets were resuspended in 6× sample buffer. Volumes corresponding to 100 or 5 ng CA were then analyzed by Western Blotting with antibodies against Nef and CA, respectively.

Flow cytometry

1×10^6 Jurkat T cells were infected in culture medium containing HEPES (pH7.4, 20 mM) and DEAE-dextran (20 μg/ml) with HIV-1 expressing Nef variants at a concentration of 50 ng of CA/ml for 2 h at 37 °C. Cells were washed and seeded in 24-well plates. Jurkat cells were harvested 18 h and 48 h post-infection and stained with anti-CA (KC57-PE, Coulter, Miami, FL) antibody in combination with either anti-MHC-I (W6/32-FITC, Sigma-Aldricht, St Louis, MO), or anti-CD4 (CD4-APC, Immunotech-Coulter, Marseilles, France) antibodies. Briefly, cell surface molecules (MHC-I, CD4) were stained at 4 °C for 30 min prior to fixation (4% PFA for 10 min at 25 °C) and permeabilization (PBS containing 1% BSA and 0.05% saponine for 15 min at 25 °C). Intracellular staining (anti-Gag) was then performed in permeabilization medium for 45 min at 25 °C. T1 cells were fixed and stained with the HLA-A2 specific antibody BB7.2 (BD Biosciences) without prior permeabilization 5 days post-infection. Samples were analyzed by flow cytometry using a FACSCalibur cytometer (Becton Dickinson, San Jose, CA).

Immunofluorescence

To visualize the subcellular localization of Nef, TZM cells were seeded onto glass coverslips and infected with virus corresponding to 2 ng CA or transfected with 1 μg of proviral DNA or Nef.GFP expression plasmid. 36 h later, the cells were fixed with 3% paraformaldehyde/phosphate-buffered saline (10 min at room temperature), permeabilized with 0.1% Tx-100 and stained with antibodies against CA (CA1) and Nef (mAb #158 (Fackler et al., 1997)) followed by appropriate fluorescent secondary antibodies. Following extensive washing, cells were mounted with Histoprime (Histogel, Linaris, Germany). Fluorescence microscopy images were acquired with an Olympus IX70 microscope with a 60× oil immersion objective equipped with a CCD camera and processed with analySIS 3.0 software (Soft Imaging System, Münster, Germany). Final images were processed in Photoshop 6.0 (Adobe Systems, Mountain View, CA).

Replication in primary human peripheral blood lymphocytes (PBL)

To analyze the effects of Nef on HIV replication in primary human T lymphocytes, peripheral blood mononuclear cells were isolated from healthy donors by Ficoll gradients using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). For infection, cells were thawed and kept in bulk cultures in RPMI, 10% FCS at 1×10^6 cells/ml overnight. 1×10^5 cells/well were then seeded in V-bottom 96-well plates and infected with 1 ng CA virus input per well the following day. 3 days later, the cells were washed and stimulated with 2 μg/ml PHA (Sigma) and 20 nM IL-2 (Chiron, Emeryville, CA) for 3 days. After stimulation, the PBL were washed and resuspended in 200 μl RPMI containing FCS and IL-2. Each day, 100 μl cell culture supernatant was replaced with fresh medium, and the

amounts of CA in the cell culture supernatant were quantified to monitor virus replication.

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